THE 44 KD PIM-1 KINASE PHOSPHORYLATES BCRP/ABCG2 AND THEREBY PROMOTES ITS MULTIMERIZATION AND DRUG RESISTANT ACTIVITY IN HUMAN PROSTATE CANCER CELLS

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We previously showed that the 44 kD serine/threonine kinase Pim-1 (Pim-1L) can protect prostate cancer cells from apoptosis induced by chemotherapeutic drugs (Oncogene, 25: 70-78, 2006). To further explore the mechanisms of Pim-1L mediated resistance to chemotherapeutic drugs in prostate cancer cells, we employed a yeast two-hybrid screening to identify cellular proteins that are associated with Pim-1L and we found the ABC transporter BCRP/ABCG2 as one of the potential interacting partners of Pim-1L. We also showed that the expression level of Pim-1L and BCRP are up-regulated in mitoxantrone and docetaxel-resistant prostate cancer cell lines. Pim-1L is colocalized with BCRP on the plasma membrane and induces phosphorylation of BCRP at Threonine 362. Knocking-down Pim-1L expression in the drug resistant prostate cancer cells abolished multimer formation of endogenous BCRP and resensitized the resistant cells to chemotherapeutic drugs, suggesting that BCRP phosphorylation induced by Pim-1L is essential for its functionality. This is further corroborated by our finding that the plasma membrane localization and drug resistant activity of BCRP was compromised by T362A mutation. Our data suggest that Pim-1L may protect prostate cancer cells from apoptosis, at least in part, through regulation of transmembrane drug efflux pump. These findings may provide a potential therapeutic approach by disrupting Pim-1L signaling to reverse BCRP-mediated multidrug resistance.

The proto-oncogene pim-1 encodes two serine/threonine kinases with molecular weights of 33 kD and 44 kD by utilizing two alternative translation sites (1,2). These two Pim-1 proteins exhibit comparable in vitro kinase activity. However, the 44 kD Pim-1 appears to be more stable (1). Studies have shown that the 33 kD Pim-1 is monomeric in vivo, whereas the 44 kD Pim-1 is found in a complex (1), suggesting the latter may interact with more protein partners. Our current knowledge on Pim-1 kinases is largely derived from study on the 33 kDa Isoform. The 33 kD Pim-1 has been implicated in regulation of cell cycle and transcription by phosphorylating a number of substrates such as cdc25A, HP1 and p100 (3-6). Moreover, it has been shown that Pim-1 may play a role in the regulation of the survival signaling by phosphorylating BAD (7). Pim-1 is thought to play an integral role in the development of a number of human cancers, such as hematolymphoid malignancies (8,9). A number of studies demonstrated that Pim-1 is upregulated in both human prostate cancer as well as animal models and may play an important role in prostate cancer development and progression. (10-12). Pim-1 has emerged as a potential diagnostic marker in prostate cancer (10). Recently, we have shown that the 44 kD isoform Pim-1L may play a more prominent role in anti-apoptosis signaling in response to chemotherapeutic drugs in prostate cancer cells (2). The localization of the 44 kD Pim-1L is primarily on the plasma membrane and it contains an N-terminal proline-rich motif and interacts directly with tyrosine kinase Etk through an interaction between the PxXP motif and the
SH3 domain of Etk. Such interaction competes with tumor suppressor p53 for binding to Etk and activates Etk kinase activity (13).

Advanced metastatic prostate cancer treated by hormone manipulation or orchiectomy frequently leads to the development of progressive hormone-refractory prostate cancer and highly chemoresistant tumors. Several biochemical mechanisms of drug resistance have been identified in prostate cancer cell lines, including alterations of glutathione metabolism, altered topoisomerase activity and up-regulation of the transmembrane drug efflux pumps (14), and in particular ATP binding cassette (ABC) transporter family members such as multidrug resistance protein-1(MDR1/Pgp/ABCB1) and multidrug resistance associated protein 1(MRP1/ABCC1). Recently it has been reported that MRP1 but not MDR1 overexpression contributes to acquired drug resistance in two prostate cancer cell lines derived from PC3 and DU145 (14). It has been shown that the presence of a half ABC transporter, breast cancer resistance protein BCRP/ABCG2 isolates the putative prostate stem cells from the prostate tissue microenvironment through constitutive efflux of androgen and protects the putative tumor stem cells from androgen deprivation, hypoxia, or adjuvant chemotherapy, and providing the nidus for recurrent prostate cancer (15). In addition to androgen, a large set of BCRP substrates have been identified including chemotherapeutic agents, fluorescent dyes, as well as chemical toxicants (16). BCRP overexpression has been detected in a variety of mitoxantrone or other chemotherapeutic agents selected cell lines (17,18). The molecular mechanism of BCRP in drug efflux has been well studied in breast cancer cells. Several immunohistochemical studies using monoclonal and polyclonal antibodies have confirmed that BCRP is mainly localized to the plasma membrane of mammalian cells. Recent studies suggest that BCRP may function as a homodimer (19) or homotetramer (20). However, whether BCRP is involved in the multidrug resistance in prostate cancer remains elusive.

To further explore the mechanisms of Pim-1L mediated resistance to chemotherapeutic drugs in prostate cancer cells, we employed the yeast two-hybrid screening to identify cellular proteins that are associated with 44 kD Pim-1L and we found BCRP as one of the potential interacting partners of Pim-1L. We also showed that expression levels of Pim-1L and BCRP are up-regulated in mitoxantrone and docetaxel resistant prostate cancer cell lines. Pim-1L can directly interact with and phosphorylate BCRP, which promotes BCRP dimerization and ultimately its plasma membrane localization. Our data suggest that Pim-1L may protect prostate cancer cells from apoptosis, at least in part, through interacting with and phosphorylating BCRP. These findings may provide a potential therapeutic approach by disrupting Pim-1 signaling to reverse BCRP-mediated multidrug resistance.

**EXPERIMENTAL PROCEDURES**

*Yeast two-hybrid screening*- The full-length 44 kD human Pim-1 cDNA was amplified by PCR using kinase inactive mutant plasmid described previously (2) as template with high fidelity polymerase(GIBCO/BRL) and primers 5’ CGGAATTCCTAGCCTCCTGCCCCGCGGCG 3’ and 5’CGGAATTCCTATTTGCTGGGCCCCGGCGAC 3’. The products were digested with EcoRI and inserted into the pGBK7T vector (Clontech). The expression library was the human HeLa cell cDNA library (Clontech). Plasmids were introduced into yeast strain Y187 and interacting protein were double selected for growth on His/Leu/Trp deficient plates and β-galactosidase production. DNA sequencing was obtained using an automated sequencing apparatus (Applied Biosystems).

*Plasmid constructs*- The human BCRP cDNA was amplified by PCR using a BCRP construct (19) as template and then subcloned into the HA and Myc tagged expression vectors respectively to generate the N-terminal tagged HA-BCRP and Myc-BCRP construct. All human Pim-1 constructs contain the N-terminal Flag-tag and described previously (2). To generate the BCRP T362A or T362D mutant, the threonine residue at position 362 was mutated to alanine or aspartic acid via oligonucleotide-directed mutagenesis with the sense primers 5’ GAAGAAGATCGCAGTCTTCAAGG 3’ or 5’GAAGAAGATCGACGTCTTCAAGG 3’ and their complementary antisense primers by using the Quickchange Mutagenesis Kit (Stratagene). GST-Pim-1L and GST-proline-rich region of Pim-1L (GST-PR) constructs were generated by PCR subcloning into pGEX-6P
vector (Amersham Pharmacia biotech) with the forward primer 5' CGGAATTCTAGCCTCC TGCCCCGCGCGG 3' with the reverse primers 5' CGGAATTCTATTGTGGCCCGCGAC 3' and 5' CGGAATTCTACCCAGCTCCGAG GTG 3' respectively. GST-Pim-1S construct was described previously (21).

Cell culture and transfection- CWR-R1 cells were kindly provided by Dr. CW Gregory (22). All other cell lines used in this study were purchased from American Tissue Culture Collections. The 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum. LNCaP cells were maintained in RPMI1640 supplemented with 10% fetal bovine serum. CWR-R1 cells were maintained in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum. Transfections were performed by using Fugene 6 (Roche), Lipofectamine 2000 (GIBCO/BRL) or the calcium phosphate precipitation method (Biological Mimetics Inc.) according to the manufacturer’s instructions.

GST-pull-down assay- GST fusion proteins were expressed and purified as described previously (13,23). Briefly, the GST fusion proteins were pulled down by glutathione beads at 4°C for 1 h and then washed three times with the lysis buffer (21,24). The immobilized GST fusion proteins were incubated with the lysates of 293T cells transfected with the HA-tagged BCRP for 1 h at 4°C. The beads were washed with the lysis buffer four times and then the protein complexes were loaded in SDS–polyacrylamide gel electrophoresis (PAGE), followed by immunoblotting with anti-HA antibody.

Immunoprecipitation, compartmental protein extraction, Western blot and in vitro kinase assay- The transfected cells were lysed in the buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na2VO4, 1 mg/ml apronin, 1 mg/ml leupetin and 1 mM PMSF). Insoluble material was removed by centrifugation, and antibodies were added to lysates and incubated for 1–3 h at 4°C. The immunocomplexes were collected by using protein A or protein G–sepharose beads, and the beads were then washed extensively for three times at 4°C with the lysis buffer. Cell fractionation was carried out using compartmental protein extraction kit (BioChain, Inc.). Immunoblotting was performed as described previously (24). Briefly, blots were incubated with primary antibodies, 1:1000 dilution of anti-Myc tag, 1:500 dilution of anti-phosphothreonine, 1:2000 dilution of anti-Flag tag, 1:2000 dilution of anti-HA tag, 1:100 dilution of anti-Pim1, 1:500 dilution of anti-BCRP(BXP21) at room temperature for 1 h, and followed by detection with horseradish peroxidase-conjugated secondary antibody. The polyclonal phospho-specific antibody for BCRPT362 (anti-pBCRP) was developed by immunizing the rabbits with a peptide containing phosphothreonine 362 of BCRP, and the terminal bleeds were affinity purified using Sepharose-immobilized peptide by a commercial carrier (Bethyl). The polyclonal anti-Pim-1L antibody was described previously (2) and the monoclonal anti-Pim-1 (cat# sc13513) was purchased from Santa Cruz. The Pim-1L IVK assays were carried out as described previously (2,25). Briefly, the purified kinase active GST-Pim-1L or kinase-inactive Pim-1LKM protein was mixed with immunoprecipitated HA-BCRP and incubated at room temperature in kinase buffer (50 mM HEPEs, pH 7.4, 10 mM MgCl2, 1 mM DTT and 200 µM ATP) for 30 min. The reaction was terminated by adding the equal volume of 2×SDS sample buffer. The phosphorylation of BCRP was detected by Western blot with anti-pBCRP362 antibody.

Immunofluorescence staining and microscopy- LNCaP cells were seeded on coverslips coated with poly-D-Lysine and transfected with 0.5 µg DNA/105 cells by the Lipofectamine 2000 transfection reagent (GIBCO/BRL). At 48 h post-transfection, the cells were fixed in 3.7% paraformaldehyde for 15 min. Immunostaining was performed by incubating the slides with 1:200 dilution of anti-Flag monoclonal antibody (M2) for 45 min and/or with 1:200 dilution of anti-HA for 1 h at room temperature, followed by incubation with the Rhodamine-conjugated goat anti-mouse antibody and the FITC-conjugated goat anti-rabbit antibody for 15 min. Immunostaining was performed by incubating the slides with 1:200 dilution of anti-Flag monoclonal antibody (M2) for 45 min and/or with 1:200 dilution of anti-HA for 1 h at room temperature, followed by incubation with the Rhodamine-conjugated goat anti-mouse antibody and the FITC-conjugated goat anti-rabbit antibody for 45 min at room temperature. The slides were then washed and mounted with Vectashield (Vector Laboratories). The stained slides of BCRP mutant T362A were examined by using an inverted fluorescence microscope under a 60X oil-immersion objective and the co-localization of wild type BCRP with Pim-1L were scanned with a laser confocal system.

Drug treatment and drug-sensitivity assay- Drug resistant cell lines R1/MX, R1/DTX were
established from the CWR-R1 cell line by culturing the cells in the medium containing mitoxantrone (MX) (Sigma) or docetaxel (DTX) (kindly provided by Sanofi Aventis). The initial concentration for selection was determined by treating cells with 0.1, 1, 2, 5 and 10nM MX or DTX for 7 days. Based on the cell survival rate (about 50%) after treatment for 7 days, 5 nM MX or 1nM DTX was used as the starting concentration. The drug resistant cells were selected by stepwise increasing drug concentration. In 3 months, the cells could grow in the presence of 20nM MX and 5nM DTX respectively and they were then maintained under these conditions. For drug sensitivity assay, LNCaP or CWR-R1 cells were infected with the lentiviruses encoding the proteins or siRNA as indicated by using the protocol described previously (2,13). The siRNA target sequence of human \textit{pim-1} gene is GCAGGACAGTGCTTGATAC and \textit{BCRP} gene is GCACAGCAAATGCTGTCCT. The direct complementary sequences were used as the scrambled siRNA control for each target: CGTCTGTCAACGAATATGC (\textit{pim-1}), CGTGTCGTTACACGAA (\textit{BCRP}). We were able to achieve over 95% infection rate routinely as determined by expression of the GFP marker. At 48 h post-infection, the cells were seeded into the 96-well plates (3 X 10^4/well). Mitoxantrone, toptecan or docetaxel was added in the medium after 24 h. The effects of these drugs on the viability of cells were measured after 20–48 h by the WST-1 assay (Roche Molecular Biochemicals). The relative viability was expressed as the mean ± SD of the triplicate for the drug-treated samples relative to the untreated controls. An aliquot of cells was lysed and followed by immunoblotting with anti-HA or anti-Flag, anti-BCRP to monitor the transfection efficiency.

**RESULTS**

Among 16 positive clones identified in our yeast two-hybrid screening using the kinase-inactive Pim-1L mutant as bait, two independent clones harbored in-frame DNA sequence coding for BCRP. This interaction appears to occur in mammalian cells because BCRP was co-immunoprecipitated with Pim-1L but not Pim-1S which lacks the N-terminal proline-rich region (Figure 1A), suggesting that BCRP preferentially binds to Pim-1L. The GST-pull-down experiments showed that BCRP was associated with GST-Pim-1L but not with GST, GST-Pim-1S, or GST-PR (Figure 1B), suggesting that only Pim-1L is able to directly interact with BCRP and this interaction requires both the N-terminal proline-rich region and the kinase domain of Pim-1L. We also observed the co-localization of BCRP and Pim-1L on the plasma membrane (Figure 1C). Furthermore, the interaction between endogenous BCRP and Pim-1L in a prostate cancer cell line CWR-R1 was confirmed by the co-immunoprecipitation experiments shown in Figure 1D.

To determine whether the interaction of Pim-1L and BCRP is involved in drug response, we treated CWR-R1 cells with lentiviruses encoding the siRNAs specific for Pim-1, BCRP or together and then tested their response to chemotherapeutic drugs. As shown in Figure 2A, knocking down Pim-1 or BCRP sensitizes CWR-R1 cells to mitoxantrone (MX) and docetaxel (DTX) compared to the vector controls. These data suggest that both Pim-1 and BCRP are required for survival of CWR-R1 cells in response to these drugs. In addition, we established two drug resistant cell lines R1/MX and R1/DTX from an AR-positive hormone refractory prostate cancer cell line CWR-R1 by culturing them in media containing MX or DTX, two FDA approved drugs for treatment of advanced hormone refractory prostate cancer. As shown in Figure 2B, both R1/DTX and R1/MX cells demonstrated increased resistance to multiple drugs including MX, DTX and TPT compared to the parental cells. The expression level of BCRP and Pim-1L was also increased in these drug resistant cell lines compared to the parental line. Meanwhile, we did not detect an appreciable increase of MDR1 in these resistant lines (Figure 2C), suggesting that BCRP may play a major role in the drug resistance in these cell lines.

To further examine if BCRP and Pim-1L act together to protect cells, we infected LNCaP cells with lentiviruses encoding the HA-tagged BCRP and/or the Flag-tagged Pim-1L and then examined their effects on drug response. LNCaP cells express very little BCRP and are very sensitive to MX or DTX. However, overexpression of either BCRP or Pim-1L alone in LNCaP cells could increase survival to some degree as previously reported and co-expression of Pim-1L and BCRP
led to a dramatic increase in cell viability (Figure 2D). These data suggest that an additive effect of BCRP and Pim-1L in anti-apoptosis signaling against chemotherapeutic drugs and the drug resistant activity of BCRP could be enhanced by the elevated Pim-1L activity in these cells.

As shown in Figure 3A, several identified Pim-1 substrates contain the consensus sequence of the preferred substrates of Pim-1: (K/R)3XS/TX (where X stands for any residue). Interestingly, the threonine 362 (T362) residue of BCRP is embedded in the similar sequence context, suggesting that BCRP could serve as a substrate for Pim-1L. This is supported by the observation that the immunoprecipitated BCRP from R1/MX and R1/DTX cells could be recognized by a phospho-threonine specific antibody (Figure 3B). Co-expression of BCRP and Pim-1L in 293T cells induces threonine phosphorylation of BCRP and substitution of T362 with alanine (T362A) or the phosphorylation-mimicking aspartate (T362D) completely abolished threonine phosphorylation of BCRP, suggesting that T362 may indeed be phosphorylated by Pim-1L in vivo (Figure 3C). To further characterize phosphorylation of BCRP T362, we developed a polyclonal antibody that specifically recognizes phosphorylated T362 of BCRP (anti-pT362). As shown in Figure 3D, this antibody recognized the wild-type BCRP phosphorylated by Pim-1L but not the unphosphorylatable T362A mutant. In addition, the anti-pT362 antibody detected the phosphorylated endogenous BCRP in R1/DTX and R1/MX cells and this phosphorylation was reduced when Pim-1L was knocked down by the siRNA (Figure 3E). Furthermore, our in vitro kinase assays demonstrated that purified GST-Pim-1L, but not the kinase-inactive mutant Pim-1LKM, can directly phosphorylate BCRP in vitro (Figure 3F). These data suggest that BCRP is threonine phosphorylated and Pim-1L may be responsible, at least in part, for phosphorylating BCRP in these cells. The requirement of both BCRP and Pim-1L for their drug resistance was further supported by that down-regulation of either BCRP or Pim-1L by the specific siRNAs resensitizes these cells to drugs (Figure 3G).

To test whether phosphorylation of T362 of BCRP is important for BCRP-mediated drug resistance, we infected LNCaP cells with the lentivirus encoding the HA-tagged BCRP or the T362A mutant and then examined whether LNCaP cells were protected by overexpression of BCRP or its mutant from apoptosis induced by chemotherapeutic drugs MX, TPT and DTX. As shown in Figure 4A, the wild-type BCRP significantly increased the survival of LNCaP cells in response to these drugs, but the T362A mutant failed to do so, suggesting that the integrity of T362 is essential for the functionality of BCRP. Co-expression of BCRP and Pim-1L in LNCaP cells exerted an additive effect on protecting LNCaP cells from apoptosis which was significantly diminished when T362 was mutated into alanine. In addition, the BCRP drug resistant activity was significantly reduced when endogenous Pim-1L expression was knocked down by the siRNA while the phosphorylation-mimicking mutant T362D remained active independent of Pim-1L (Figure 4B). These results suggest that phosphorylation of BCRP T362 induced by Pim-1L may be required for its optimal efflux activity.

Based on the topological structure of BCRP, T362 localizes in the linker region between the ATP binding pocket and the transmembrane domain. To test if T362 phosphorylation plays a role for BCRP membrane localization, we infected LNCaP cells by the lentivirus encoding the wild-type BCRP and the T362A mutant. Immunofluorescence microscopy revealed that the wild-type BCRP and the phosphorylation-mimicking mutant T362D are predominantly localized on plasma membrane, while the T362A mutant is mainly localized in cytoplasmic compartment (Figure 5A). The retention of BCRP in the cytoplasmic compartment caused by the T362A mutation was further confirmed by fractionation experiments (Figure 5B). It has been reported that the dimerization/oligomerization of BCRP on the plasma membrane plays a critical role for its drug efflux activity (19). We therefore examined the effects of T362A mutation on BCRP dimerization. We co-transfected the wild-type HA-tagged BCRP with the Myc-tagged BCRP or its T362A mutant into 293T cells. The status of BCRP dimerization/oligomerization was examined by the co-immunoprecipitation assays. As shown in Figure 5C, the wild-type Myc-BCRP was efficiently associated with the wild-type HA-BCRP but such interaction was disrupted by the T362A mutation. This is further corroborated by
our observation that the formation of endogenous BCRP multimers in R1/MX cells was compromised when Pim-1L expression was knocked down by the specific siRNA (Figure 5D). Taken together, these data suggest that phosphorylation of BCRP T362 induced by Pim-1L may modulate the dimerization/oligomerization of BCRP and its plasma membrane presentation.

DISCUSSION

Previously we have shown that the 44 kD Pim-1 kinase (Pim-1L) predominantly localizes on plasma membrane and plays a more prominent role than the 33 kD isoform (Pim-1S) in anti-apoptosis signaling in response to chemotherapeutic drugs in prostate cancer cells. To identify the potential Pim-1L specific substrates, we carried out the yeast two-hybrid screening. Interestingly, none of the preys identified in our survey was previously reported as Pim-1S-associated proteins by using the similar screening approach. This suggests that Pim-1L and Pim-1S may interact with a different pool of protein partners and therefore play distinct roles in cell signaling. Further study on the Pim-1L interacting and signaling network would reveal the mechanisms by which Pim-1L exerts its biological activity. In this study, we provided the evidence that BCRP/ABCG2 is a preferential substrate of Pim-1L and is an important mediator for Pim-1L promoted drug resistance in prostate cancer cells. It appears to require both the N-terminal proline-rich domain and the kinase domain for Pim-1L to interact with BCRP, suggesting that more than one interacting sites may be present between BCRP and Pim-1L. We also showed that phosphorylation of BCRP T362 by Pim-1L modulates the dimerization/oligomerization of BCRP and its plasma membrane localization. Previous in vitro studies showed that BCRP may form homodimers through disulfide bond mediated by Cysteine 603, however, such covalent intramolecular link does not appear to be prerequisite for BCRP to exert its transport activity (26,27). It remains to be investigated how phosphorylation of T362 affects the intermolecular interaction between BCRP dimers or multimers. It is possible that phosphorylation of this site would induce a conformational change of BCRP and promote the nearby highly conserved Cys374 to form intermolecular disulfide bond. Additional experiments have to be done to test whether it is indeed the case. We previously showed that Pim-1L can interact with tyrosine kinase Etk on the plasma membrane and Etk is known to be involved in regulation of vesicle trafficking and promotes plasma membrane presentation of EGFR (13,24). It is possible that BCRP could be regulated by both kinases in a dynamic fashion. Future investigations are needed to elucidate how these proteins work together to confer drug resistance.

A previous study showed that overexpression of BCRP in breast cancer cells does not confer resistance to paclitaxel (9). However, in this study, we showed the evidence that BCRP is involved in resistance to docetaxel, a taxol derivative, in prostate cancer cells, suggesting that BCRP may be able to transport a broader range of substrates than we previously thought depending on cellular context. It is not yet clear whether phosphorylation of BCRP induced by Pim-1L may alter the substrate spectrum of the transporter. BCRP has been shown to be highly expressed in a subset of prostate cancer stem/progenitor cells. It would be interesting to examine whether the drug resistant lines we established in this study may possess the property of cancer stem/progenitor lines. To further investigate whether BCRP may play a role in docetaxel resistance in human prostate cancer, we conducted a survey on tissue samples from patients who were treated with docetaxel for at least six months using our anti-pT362 antibody. Our preliminary analysis revealed a significant increase of BCRPT362 phosphorylation in the docetaxel resistant samples compared to the untreated control groups, suggesting that BCRP activity may contribute to docetaxel resistance in prostate cancer. It remains to be tested whether the anti-pT362 antibody could be used to predict patient outcome in response to chemotherapy. Given that down-regulation of BCRP and Pim-1L expression sensitize prostate cancer cells to chemotherapeutic drugs, it is conceivable that combination treatment with Pim-1 or BCRP inhibitors would significantly enhance the efficacy of conventional chemotherapy for prostate cancer and prevent the development of drug resistance.

REFERENCES

FOOTNOTES
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FIGURE LEGENDS

Fig. 1. Interaction between Pim-1L and BCRP. A. Association of Pim-1L with BCRP in vivo. 293T cells were transfected with the plasmids indicated. Immunoprecipitation was carried out using the anti-Flag and followed by immunoblotting with anti-HA. B. Direct interaction between BCRP and Pim-1L. The GST-pull-down experiments were carried out by incubation of the immobilized GST-Pim-1L, GST-Pim-1S, GST-proline-rich region of Pim-1L (GST-PR) fusion protein or the GST control with the cell lysates from 293T cells overexpressing HA-BCRP. C. Colocalization of Pim-1L and BCRP. LNCaP cells were transfected with Flag-tagged Pim-1L and HA-BCRP. BCRP protein was detected by staining with anti-HA and Rhodamine-labeled secondary antibodies (red). Pim-1L protein was detected by staining with polyclonal anti-Pim-1L and FITC-labeled secondary antibodies (green). Yellow indicates the colocalization of BCRP and Pim-1L. D. Endogenous BCRP is associated with Pim-1L in CWR-R1 cells. Lysates from CWR-R1 cells were immunoprecipitated with the polyclonal antibody specific for Pim-1L or the preimmune serum control, followed by immunoblotting with anti-BCRP or anti-Pim-1L. The amount of Pim-1L and BCRP in the total cell lysates (TCL) was monitored by Western blot.

Fig. 2. Pim-1L promotes BCRP mediated drug resistance. A. Downregulation of Pim-1L or BCRP expression sensitizes CWR-R1 cells to chemotherapeutic drugs. CWR-R1 cells were treated with the lentiviruses encoding the siRNA for Pim-1 with or without the siRNA for BCRP, or the vector control. At 48 h post-infection, these cells were plated in 96-well plates and subjected to treatment of mitoxantrone (MX) (5 µM) or docetaxel (DTX) (1µM) for 48 h. *P<0.01 compared with the vector control. B. Multidrug resistance of R1/MX and R1/DTX. R1/MX, R1/DTX and their parental cells (R1) were subjected to treatment of MX (5µM), DTX (0.1µM) or toptecan (TPT) (2 µM) for 48 h and the viability of these cells was determined by WST-1 assays. *P<0.01 compared with the vector control. C. Up-regulation of Pim-1L and BCRP in prostate cancer cells. The cell lysates from the parental CWR-R1 and DTX (5nM), MX (10nM) resistant cells were subjected to immunoblotting with anti-Pim1, anti-BCRP and anti-MDR1 (top) respectively. D. Pim-1L promotes BCRP mediated drug resistance. LNCaP cells were treated with the lentiviruses encoding the Pim-1L with or without lentivirus encoding the BCRP, or the vector control. At 48 h post-infection, these cells were subjected to treatment of MX or DTX for 48 h and the viability of these cells was determined by WST-1 assays. The Student t-Test was performed. *P<0.01 compared with the vector control.

Fig. 3. Pim-1L induce BCRP phosphorylation at Threonine 362. A. Alignments of phosphorylation consensus sequences from identified Pim-1 kinase substrates. B. Endogenous BCRP in prostate cancer cells is threonine phosphorylated. Lysates from CWR-R1 parental and R1/MX, R1/DTX cells were immunoprecipitated with anti-BCRP or IgG control, followed by immunoblotting with anti-phosphothreonine (pThr) or anti-BCRP. C. Pim-1L phosphorylates BCRP. 293T cells were transfected with the plasmids as indicated. BCRP phosphorylation was examined as in 1A . D. Pim-1L phosphorylates BCRP at Thr 362. 293T cells were transfected with the plasmids as indicated. At 24 h post-transfection, the cells were serum starved overnight. The cell lysates were subjected to immunoprecipitation with anti-HA antibody and followed by immunoblotting with the anti-phosphothreonine 362 (pT362). E. Downregulation of Pim-1L decreases endogenous BCRP phosphorylation at Thr362. CWR-R1/DTX cells were treated with the lentivirus encoding the siRNA for Pim-1 or the vector control. The endogenous BCRP threonine phosphorylation was determined by...
immunoprecipitation with anti-BCRP, followed by immunoblotting with the anti-phosphothreonine (pThr362). F. Pim-1L phosphorylates BCRP at T362 in vitro. The in vitro kinase assays were carried out as described in Materials and Methods. The phosphorylation of BCRP was detected by immunoblotting with anti-pBCRPT362 antibody. The amount of GST-Pim-1L and BCRP in the reactions were monitored by immunoblotting with anti-Pim-1 and anti-BCRP repectively. G. Resensitization of drug resistant cells by downregulation of BCRP or Pim-1L. R1/MX, R1/DTX cells were treated with the lentivirus encoding the siRNA for Pim-1 or BCRP, or the vector or the scrambled siRNA control. At 48h post-infection, the cells were maintained in MX or DTX containing medium for 72 h. Cell growth was determined by WST-1. *P<0.01 compared with the vector control. Meanwhile, the cell colonies were visualized by Coomassie blue staining.

Fig. 4. Pim-1L increases BCRP mediated drug resistance through phosphorylation of BCRP at T362. A. The effects of BCRP phosphorylation site mutant T362A on drug resistant activity. LNCaP cells were treated with the lentivirus encoding the Pim-1L and/or BCRP as indicated. At 48 h post-infection, the cells were subjected to treatment of MX(1μM), TPT(2μM) or DTX(10μM) for 48 h. The viability of these cells was determined by WST-1 assays. *P<0.01 compared with the vector control. B. T362D mutant mediated drug resistance is independent of Pim-1L. LNCaP cells were treated with the lentivirus encoding BCRP alone or with the lentivirus encoding the siRNA for Pim-1. At 48 h post-infection, the cells were subjected to treatment of MX(0.5μM) or TPT(2μM) for 48 h followed by WST-1 assays. *P<0.01 compared with the vector control.

Fig. 5. Effects of T362A mutation on the subcellular distribution and multimerization of BCRP in PCA cells. A. T362A mutant predominately localizes in the intracellular compartment. LNCaP cells were treated with the lentivirus encoding the wild-type BCRP, T362A or T362D mutant. BCRP was detected by staining with anti-HA and Rhodamine-labeled secondary antibodies (red). Cells were also stained with DAPI to identify the nuclear compartment. The stained slides were examined by using an inverted fluorescence microscope. B. Subcellular distribution of BCRP. LNCaP cells were treated with the lentivirus encoding the wild-type BCRP or T362A mutant, or the vector control. Cells were then fractionated as described in Materials and Methods. The expression of BCRP was determined by immunoblotting with anti-HA. The blot was then probed with anti-actin to monitor the sample loading and anti-Hsp90, anti-EGFR to assign the majorities of the marker proteins to their expected cytoplasmic, membrane fractions respectively. C. T362A mutation disrupts the dimerization of BCRP. 293T cells were transfected by the plasmids as indicated. At 24 h post-transfection, the cell lysates were subjected to immunoprecipitation with anti-HA and followed by immunoblotting with anti-Myc. D. Effects of Pim-1 siRNA on oligomerization of the endogenous BCRP in R1/MX cells. R1/MX cells were treated with the lentivirus encoding the siRNA for Pim-1 or vector alone. At 48 h post-infection, the cells were collected and the lysates were mixed with 2XSDS loading buffer with β-mercaptoethanol(β-ME) or without β-ME. The expression of BCRP or Pim-1 was determined by immunoblotting with anti-BCRP or anti-Pim-1.
Figure 1

A

<table>
<thead>
<tr>
<th>Pim-1L+</th>
<th>Pim-1S+</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCRP</td>
<td>BCRP</td>
</tr>
<tr>
<td>Vector</td>
<td>Vector</td>
</tr>
<tr>
<td>IP: α Flag</td>
<td>IB: α HA</td>
</tr>
<tr>
<td>IB: α HA</td>
<td>IB: α HA</td>
</tr>
<tr>
<td>IB: α Flag</td>
<td>IB: α Flag</td>
</tr>
</tbody>
</table>

B

IB: α HA

Coomassie Blue

IB: α HA (Input)

C

Anti-HA  Anti-Pim-1L  Merge

D

IP:

<table>
<thead>
<tr>
<th>IP</th>
<th>Preimmune</th>
<th>Pim-1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB: α BCRP</td>
<td>IB: α Pim-1L</td>
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</tr>
</tbody>
</table>

TCL

| IB: α BCRP | IB: α Pim-1L |

Figure 2

A

![Graph showing relative viability (%) for Vector, SiPim-1, SiBCRP, and SiPim-1+SiBCRP in MX and DTX treatments.](image)

B

![Graph showing relative viability (%) for R1, R1/MX, and R1/DTX in DTX, MX, and TPT treatments.](image)

C

![Western blot analysis for IB: α Pim-1, IB: α BCRP, IB: α MDR1, and IB: α Actin in MX and DTX treatments.](image)

D

![Graph showing relative viability (%) for MX and DTX treatments with Vector, BCRP, Pim-1L, BCRP+Pim-1L, BCRP+Pim-1S, and IB: α HA, IB: α Pim-1.](image)
Figure 3

**A**

<table>
<thead>
<tr>
<th>Protein</th>
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<td>ELRRMSEDFVDS</td>
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<tr>
<td>p21 140-151</td>
<td>RKRRQTSMTDFY</td>
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<td>LANA 200-211</td>
<td>RKRRLLSPQGPS</td>
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<td>BCRP 357-368</td>
<td>KKKKITVFKIES</td>
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**B**

<table>
<thead>
<tr>
<th>Treatment</th>
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<tbody>
<tr>
<td>R1</td>
<td>IB: α pThr</td>
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<tr>
<td></td>
<td>IB: α BCRP</td>
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<tr>
<td></td>
<td>IB: α Actin</td>
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**C**

<table>
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<tr>
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<tbody>
<tr>
<td>MycBCRP</td>
<td>IB: α pThr</td>
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<tr>
<td></td>
<td>IB: α Myc</td>
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<tr>
<td></td>
<td>IB: α Myc</td>
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<td>IB: α Flag</td>
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**D**

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<tbody>
<tr>
<td>HABCRP+</td>
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<tr>
<td></td>
<td>IB: α pT362</td>
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<tr>
<td></td>
<td>IB: α HA</td>
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<tr>
<td></td>
<td>IB: α Actin</td>
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</table>

**E**

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<tbody>
<tr>
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<tr>
<td></td>
<td>IB: α pT362</td>
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<tr>
<td></td>
<td>IB: α Pim-1</td>
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<tr>
<td></td>
<td>IB: α BCRP</td>
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<td>IB: α Actin</td>
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**F**

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<th>Western Blots</th>
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</thead>
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<td>IB: α Pim-1</td>
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<tr>
<td></td>
<td>IB: α BCRP</td>
</tr>
</tbody>
</table>

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Figure 3 (continued)

G

R1/DTX

% cell growth

Vector  SiBCRP

0  20  40  60  80  100

Vector  SiPim-1

*  *  *

Pim-1

BCRP

GAPDH

R1/MX

% cell growth

Vector  SiBCRP

0  20  40  60  80  100

Vector  SiPim-1

*  *  *

Pim-1

BCRP

GAPDH

Vector scBCRP scPim-1

siBCRP siPim-1

Vector scBCRP scPim-1

siBCRP siPim-1

R1/DTX

R1/MX
Figure 4

A

MX

TPT

Relative viability (%)

Vector
Pim-1L
Pim-1LKM
Vector
Pim-1L
Pim-1LKM

+BCRP
+T362A

Relative viability (%)

Vector
Pim-1L
Pim-1LKM
Vector
Pim-1L
Pim-1LKM

+BCRP
+T362A

IB: α HA
IB: α Flag

DTX

Relative viability (%)

Vector
Pim-1L
Pim-1LKM
Vector
Pim-1L
Pim-1LKM

+BCRP
+T362A

Relative viability (%)

Vector
Pim-1L
Pim-1LKM
Vector
Pim-1L
Pim-1LKM

+BCRP
+T362A

IB: α Flag

B

MX

TPT

Relative viability (%)

Vector
Sphm-1
Vector
Sphm-1

+BCRP
+T362D

Relative viability (%)

Vector
Sphm-1
Vector
Sphm-1

+BCRP
+T362D

IB: α Pim-1
IB: α BCRP
IB: α Actin
Figure 5

A

BCRP  BCRPT362A  BCRPT362D

B

Vector  BCRP  T362A

BCRP  Hsp90  EGFR

C

IP: α HA
IB: α Myc

IP: α HA
IB: α HA

IB: α HA

IB: α Myc

D

R1/MX

Vector  Sipim-1

- ME
+ME

IB: α BCRP

IB: α Pim-1

IB: α GAPDH

multimer

multimer

monomer
The 44 kD Pim-1 kinase phosphorylates BCRP/ABCG2 and thereby promotes its multimerization and drug resistant activity in human prostate cancer cells

Yingqiu Xie, Kexin Xu, Douglas E. Linn, Xi Yang, Zhiyong Guo, Hermela Shimelis, Takeo Nakanishi, Douglas D. Ross, Hegang Chen, Ladan Fazli, Martin E. Gleave and Yun Qiu

*J. Biol. Chem.* published online December 5, 2007

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